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Expression in *Escherichia coli* of the 37-Kilodalton Endoflagellar Sheath Protein of *Treponema pallidum* by Use of the Polymerase Chain Reaction and a T7 Expression System

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We previously reported the complete primary structure of the 37-kilodalton endoflagellar sheath protein (FlaA) of *Treponema pallidum*. However, we were unable to determine the nucleotide sequence of *flaA* upstream of amino acid 10. The desired nucleotide sequence was obtained by use of a strategy based upon the polymerase chain reaction and was found to contain a consensus *Escherichia coli* promoter, a ribosomal binding site, and a 20-amino-acid signal peptide. Expression of FlaA in *E. coli* was achieved by cloning polymerase chain reaction-derived constructs lacking the native *T. pallidum* promoter into a temperature-inducible T7 expression system. Pulse-chase and ethanol inhibition analyses of protein processing in *E. coli* cells and minicells, respectively, indicated that processing of the FlaA precursor was incomplete. Native and recombinant FlaA were identical as assessed by antibody reactivity and sodium dodecyl sulfate- and two-dimensional polyacrylamide gel electrophoretic mobilities. Soluble FlaA was not detected in either the cytoplasmic or the periplasmic fractions of *E. coli* transformants. Fractionation of *E. coli* cell envelopes unexpectedly revealed that FlaA precursor and FlaA were associated with both the cytoplasmic and outer membranes. This is the first report of expression in *E. coli* of a *T. pallidum* protein which could not be cloned or expressed with its native promoter. Our data also indicate that information obtained in *E. coli* regarding the subcellular location of cloned treponemal proteins must be cautiously extrapolated to *T. pallidum*.

Venereal syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* (*T. pallidum*). A significant increase in reported cases of syphilis in the United States has occurred in the last two years (13), adding impetus to research aimed at understanding the pathogenesis of this disease. Motility, a characteristic feature of all spirochetes (31), is thought to contribute to the ability of *T. pallidum* to invade and disseminate within mammalian hosts (71). The organelles of motility, the endoflagella (11, 33, 38, 55), are polymeric structures within the periplasmic space of the organism (32) consisting of 33- and 33.5-kilodalton (kDa) core and 37-kDa sheath subunits (8, 15, 48, 56). The endoflagellar antigens stimulate B- and T-cell responses in humans (5, 26; unpublished data) and in experimental syphilis (4, 25, 39; unpublished data), suggesting that they also may play a role in the immunopathogenesis of the disease.

Treponemal research has been hampered by the inability to cultivate *T. pallidum* continuously in vitro. Many investigators have circumvented this problem, in part, by expressing treponemal antigens in *Escherichia coli* (for a review, see reference 68). In the course of these studies, data obtained in *E. coli* regarding the cellular location and membrane topography of cloned treponemal antigens often have been extrapolated to the native *T. pallidum* proteins (14, 18, 28, 64, 68). However, to what extent *E. coli* can be used as a model for molecular analysis of *T. pallidum* proteins remains unclear. It is now recognized that these two organisms differ greatly with respect to both the protein and lipid compositions of their outer membranes and the relative antigenicities of their respective surfaces (59, 75). The periplasmic endoflagellar proteins are excellent candidates for addressing these issues as they are the only antigens of *T. pallidum* that have been localized unequivocally within the organism.

We previously reported the primary structure of the 37-kDa sheath protein of *T. pallidum* (FlaA) along with a partial nucleotide sequence of *flaA* (33). However, we were unable to clone the portion of *flaA* upstream of amino acid 10, presumably because of toxicity in *E. coli*. To determine the complete nucleotide sequence of *flaA* and to express FlaA in *E. coli*, we employed an alternative cloning strategy based upon the polymerase chain reaction (PCR) (61). This is the first report of expression in *E. coli* of a *T. pallidum* protein which could not be cloned or expressed with its native promoter. Our data also indicate that information obtained in *E. coli* regarding the subcellular location of cloned treponemal proteins must be cautiously extrapolated to *T. pallidum*.

(A preliminary report of this work has been presented (R. D. Isaacs and J. D. Radolf, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, D-160, p. 107].)

MATERIALS AND METHODS

Bacterial strains. *T. pallidum* (Nichols) was passaged by intratesticular inoculation into New Zealand White rabbits and extracted as previously described (27, 42, 58). *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and HB101 (10) were used as host strains for plasmid transformations. *E. coli* DH5αF′ (Bethesda Research Laboratories) and XL1-Blue (Stratagene, La Jolla, Calif.) were used as host strains for M13 phage (76). *E. coli* ORN103 was used for minicell analyses (50).

Plasmids and DNAs. Plasmid DNAs and oligonucleotides used in this study are listed in Table 1 and Fig. 1, respectively. *T. pallidum* chromosomal DNA was extracted as previously described (33).

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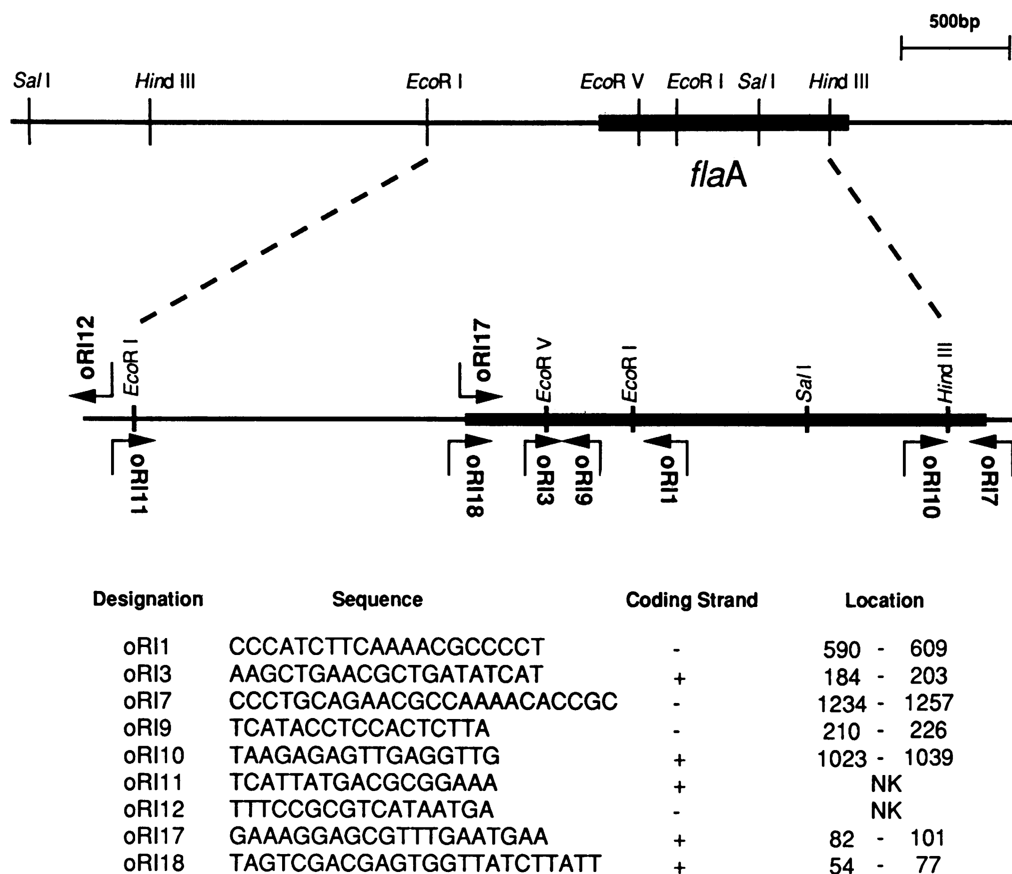

Designation	Sequence	Coding Strand	Location
oRI1	CCCATCTTCAAAACGCCCCT	-	590 - 609
oRI3	AAGCTGAACGCTGATATCAT	+	184 - 203
oRI7	CCCTGCAGAACGCCAAAACACCGC	-	1234 - 1257
oRI9	TCATACCTCCACTCTTA	-	210 - 226
oRI10	TAAGAGAGTTGAGGTTG	+	1023 - 1039
oRI11	TCATTATGACGCGGAAA	+	NK
oRI12	TTTCCGCGTCATAATGA	-	NK
oRI17	GAAAGGAGCGTTTGAATGAA	+	82 - 101
oRI18	TAGTCGACGAGTGGTTATCTTATT	+	54 - 77

FIG. 1. *T. pallidum* genetic map in the vicinity of *flaA*. The region of the chromosome between *flaA* (thick line) and the upstream *Eco*RI site has been expanded to show the locations of oligonucleotides used in these studies. The oligonucleotide sequences are 5′ to 3′, and this is indicated by the arrows. Oligonucleotides which are derived from the coding strand (+) and those that are complementary to the coding strand (−) are indicated. Nucleotide position 1 is the first nucleotide of the sequence shown in Fig. 3. The exact location of oRI11 and oRI12 is not known (NK), as their sequences were derived from a randomly selected fragment of the inverse-PCR product (see text).

SDS-PAGE and immunological reagents. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37, 58), two-dimensional PAGE (2D-PAGE) (33, 47, 49), and immunoblot analyses (33, 58) were performed as previously described. H9-2, a murine monoclonal antibody of subclass immunoglobulin G_1 specific for the 37-kDa endoflagellar sheath protein of *T. pallidum* (33, 40), was purified from hybridoma supernatants by using recombinant Protein G (Genex Corp., Gaithersburg, Md.) according to the instructions of the manufacturer. Horseradish peroxidase-conju-

TABLE 1. Plasmid DNAs used in this study

Designation	Description[a]	Source or reference
pBR322	Tc^r Ap^r, replicon pMB1	9
pUC19	Ap^r, replicon pMB1	76
pTTQ19	Ap^r, replicon pMB1	67
pBluescript II KS-	Ap^r, replicon pMB1, polylinker contains T7 promoter transcribing in opposite orientation to *lac* promoter	Stratagene, La Jolla, Calif.
pT7-3	Ap^r, replicon pMB1, *amp* is transcribed from T7 promoter	69
pGP1-2	Km^r, replicon p15A, encodes for T7 DNA-dependent RNA polymerase under control of λ promoter, temperature-sensitive λ repressor	69
pRI4	pBR322 with *T. pallidum* chromosomal DNA fragment encoding amino acids 10 through 330 of the mature *flaA* gene product	33
pRI4.3	pUC19 with 1.5-kb *Eco*RI-*Pst*I fragment from pRI4 insert	This study
pRI17	PCR product from *T. pallidum* chromosomal DNA with primers oRI17 and oRI7 digested with *Pst*I, cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI18	PCR product from *T. pallidum* chromosomal DNA with primers oRI18 and oRI7 digested with *Pst*I, cloned into pBluescript II KS- digested with *Sma*I and *Pst*I	This study
pRI19	pBluescript II KS- digested with *Bam*HI and *Pst*I, containing 0.3-kb *Bam*HI-*Eco*RI fragment of pRI18 and the 1.5-kb *Eco*RI-*Pst*I fragment from pRI4.3	This study

[a] Abbreviations: Tc^r, Ap^r, and Km^r, resistance to tetracycline, ampicillin, and kanamycin, respectively.

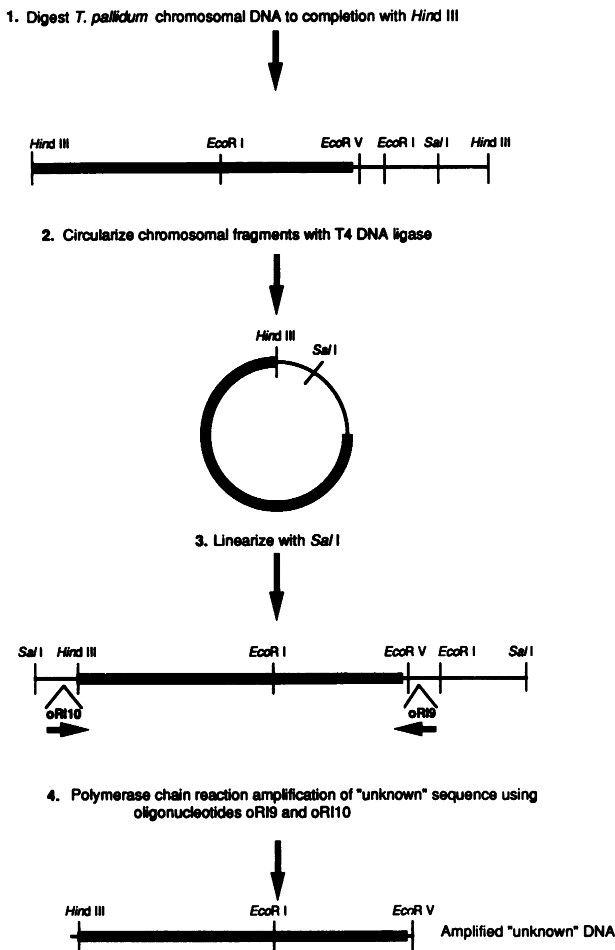

FIG. 2. Outline of the inverse-PCR procedure used to in vitro clone the upstream regions of *flaA*. The region of unknown nucleotide sequence is indicated by a thick line. The arrows under the PCR primers indicate the direction of DNA replication.

gated rabbit anti-mouse immunoglobulin G (Zymed Laboratories, San Francisco, Calif.) was used at a dilution of 1:1,000.

Isolation of *T. pallidum* endoflagella. *T. pallidum* endoflagella were isolated as previously described (33).

PCR DNA amplification. PCR was performed with 2.5 U of recombinant *Taq* polymerase (AmpliTaq; Perkin Elmer Cetus, Norwalk, Conn.) and a Perkin Elmer Cetus Thermocycler. Typically, PCRs were performed in 100-μl volumes each containing 1× PCR reaction buffer (10 mM Tris hydrochloride [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), 1 μg of each primer, 200 μM of each deoxynucleoside triphosphate, and 1 μg of chromosomal DNA. Inverse-PCR (73) was performed for 25 cycles by using oligonucleotides oRI9 and oRI10 (Fig. 2); each cycle consisted of denaturation at 94°C for 2 min, annealing at 48°C for 2 min, and extension at 72°C for 3 min. Routine and asymmetric PCRs (24, 41) with oRI1 and either oRI11 or oRI12 were performed for 25 and 40 cycles, respectively, by using the above parameters; 1 pmol of oRI1 and 20 pmol of oRI11 were used for asymmetric PCR. PCRs with oRI7 and either oRI17 or oRI18 were performed for 30 cycles, each consisting of denaturation at 94°C for 2 min, annealing at 66°C for 2 min, and extension at 72°C for 2 min.

PCR products were analyzed by ethidium bromide agarose gel electrophoresis and purified in one of the following manners: (i) from low-melting-point agarose (Sea-Plaque; FMC Corporation, Marine Colloids Div. Rockland, Maine) gels by using an Elutip-d column (Schleicher & Schuell, Inc., Keene, N.H.) (63), (ii) by electroelution from SeaKem GTG (FMC Corp.) agarose gels, or (iii) by using Centricon-30 microconcentrators (W. R. Grace & Co., Danvers, Mass.) (41). Prior to ligation reactions, the PCR product was phosphorylated by using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass).

DNA-DNA hybridizations. Double-stranded DNA probes for Southern hybridization were labeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Lisle, Ill.) by random primer labeling (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (19). Dephosphorylated oligonucleotide probes were labeled with [γ-^{32}P]ATP (Du Pont Co. Biotechnology Systems, Wilmington, Del.) by using T4 polynucleotide kinase. Colony blot and Southern blot hybridizations with double-stranded DNA probes were performed as previously described (33, 66). When oligonucleotide probes were used, prehybridization and hybridization were performed at 42°C in 6× SSC (1× SSC is 0.15 M NaCl-0.015 M sodium citrate [pH 7.0]), 50 mM sodium phosphate buffer (pH 6.8), 10% (wt/vol) dextran sulfate, and 5× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, 0.02% [wt/vol] bovine serum albumin), containing 0.1 mg of denatured, sheared, salmon sperm DNA per ml. Three washes in 6× SSC at room temperature for 10 min each were performed prior to a stringency wash at 45°C in 6× SSC-0.1% SDS for 30 min.

DNA sequence analysis. DNA sequence analysis was performed with modified T7 DNA polymerase (Sequenase 2.0; U.S. Biochemicals Corp., Cleveland, Ohio) as described previously (33, 62, 70). The University of Wisconsin Genetics Computer Group sequence analysis software package (17) was used in conjunction with Microgenie software (Beckman Instruments, Inc., Palo Alto, Calif.) for DNA sequence analysis.

Expression of FlaA. Bacteria were grown at 30°C to an A_{590} of 1.5 in LB broth containing ampicillin (40 μg/ml) and kanamycin (40 μg/ml). The temperature was shifted to 42°C for 25 min, and then rifampin was added to a final concentration of 200 μg/ml. The culture was incubated for 2 h at 30°C, and the cells were recovered by centrifugation.

Pulse-chase experiments. Bacteria were grown at 30°C to an A_{590} of 0.5 in LB broth containing ampicillin and kanamycin. Cells were recovered by centrifugation, washed in ice-cold M9 medium, and then suspended in M9 medium supplemented with 20 μg of thiamine per ml and 0.01% (wt/vol) amino acids (without methionine and cysteine). The cultures were incubated at 30°C for 30 min and then at 42°C for 15 min. Rifampin was added to a final concentration of 200 μg/ml, and the culture was incubated for an additional 10 min at 42°C. After the culture was incubated for 30 min at 30°C, Trans ^{35}S label (an approximate 80:20 mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN Biomedicals) was added; this was incubated at 30°C for 1 min. The culture was then chased with 0.1% methionine in M9 medium. Samples were removed at various times and immediately mixed with one-half the volume of ice-cold stop solution (0.04% [wt/vol] chloramphenicol, 0.4 M sodium azide, 0.02 M 2,4-dinitrophenol) (23), and the cells were recovered by centrifugation.

Minicell analyses. Bacteria were grown at 30°C for 18 h in LB broth containing ampicillin and kanamycin. Minicells were isolated as previously described (57). Minicells were suspended in M9 medium without glucose (approximately

10^8 minicells per ml) containing D-cycloserine (20 μg/ml) and incubated at 30°C for 15 min followed by incubation at 42°C for 5 min. Rifampin was added to a final concentration of 100 μg/ml, and the minicells were incubated at 42°C for an additional 5 min and then at 30°C for 15 min. Ice-cold 95% ethanol was added to each sample so that the final ethanol concentration was either 0, 1, 3, or 5% (vol/vol). After the addition of Trans ^{35}S label, each sample was incubated at 30°C for 30 min and the cells were then recovered by centrifugation.

Selective release of *E. coli* soluble periplasmic proteins. Bacteria were recovered by centrifugation at 4°C, washed in ice-cold 0.2 M Tris hydrochloride (pH 8), and then suspended in ice-cold 0.2 M Tris hydrochloride (pH 8)-1 M sucrose; EDTA and lysozyme were added sequentially to final concentrations of 0.5 mM and 60 μg/ml, respectively. An equal volume of ice-cold 0.2 M Tris hydrochloride (pH 8) was added, and the mixture was incubated on ice for 30 min. The cell pellet and the supernatant containing the soluble periplasmic fraction were separated by centrifugation at 4°C. The presence of β-lactamase in each fraction was assessed visually by using the colorimetric substrate [1-(thienyl-2-acetamido)]-3-[2-(4-*N*, *N*-dimethylaminophenylazo)pyridium methyl]-3-cepem-4-carboxylic acid (35).

Membrane fractionation experiments. Bacteria suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0) were disrupted by French press, and intact cell bodies were removed by centrifugation at 16,000 × *g* for 10 min at 4°C. Inner and outer membranes of *E. coli* were either collected by centrifugation at 240,000 × *g* for 60 min at 4°C or fractionated by using a modification of the procedure of Osborn and coworkers (51) as described by Munford et al. (44). The sucrose gradients were harvested from the top in 250-μl portions.

RESULTS

Nucleotide sequence analysis of the upstream region of *flaA*. pRI4, a chimeric plasmid isolated from a *T. pallidum* chromosomal DNA library (46), provided the starting point for the present investigations; the insert of this plasmid contains the DNA encoding for amino acids 10 through 330 of native FlaA (33). Previously, the nucleotide sequence of the upstream portion of *flaA* could not be determined because exhaustive attempts to clone this region were unsuccessful. As an alternative, we adopted a strategy based upon a variant of PCR called inverse-PCR (73). This technique enables selective amplification of a region of unknown nucleotide sequence adjacent to a region of known sequence.

It was necessary to map the *T. pallidum* chromosome in the vicinity of *flaA* prior to performing inverse-PCR. Oligonucleotide oRI3, derived from the 5' end of the coding strand of the pRI4 insert, hybridized in Southern analyses with fragments of 2.5, 2.7, and 0.9 kilobase pairs (kb) from *T. pallidum* chromosomal DNA digested to completion with *Hin*dIII, *Sal*I, or *Eco*RI, respectively (data not shown). By combining these data with the restriction map of pRI4 (33), the required genetic map was derived (Fig. 1). The previously unclonable region of *flaA* resides on a 2.5-kb *Hin*dIII genomic DNA fragment. This fragment contains *Sal*I and *Eco*RI sites in the region of known nucleotide sequence and a second *Eco*RI site in the unsequenced region (Fig. 1).

Inverse-PCR was performed as shown in Fig. 2. *T. pallidum* chromosomal DNA was digested to completion with *Hin*dIII, circularized in the presence of T4 DNA ligase, and then linearized with *Sal*I. Oligonucleotides oRI9 and oRI10, which flank the unsequenced area in the linearized chromosomal DNA, were used as the PCR primers. The resulting 1.8-kb product had an internal *Eco*RI site and hybridized with oRI3 (data not shown), confirming that the correct region had been amplified. For reasons as yet unclear, these PCR experiments worked unpredictably and yielded small amounts of product; attempts to reamplify the purified PCR product by using both routine and asymmetric PCR also were unsuccessful. Furthermore, the inverse-PCR product could not be cloned into *E. coli* by using either pUC or pTTQ vectors; pTTQ vectors contain a transcriptional stop downstream of the cloned fragment and are particularly useful for cloning strong promoters (67).

An alternative strategy was then devised for obtaining a limited amount of nucleotide sequence within the unsequenced region of the inverse-PCR product. Routine PCR could then be performed from *T. pallidum* chromosomal DNA, by using a primer derived from this new sequence, with the expectation that this new product could be more easily manipulated than the original inverse-PCR product. Southern hybridization of the inverse-PCR product digested with *Sau*3A I and probed with itself indicated that multiple sites were present (data not shown). The inverse-PCR product, digested with *Sau*3A I, was ligated into M13mp19 digested with *Bam*HI. A clone identified by DNA-DNA hybridization with the entire inverse-PCR product was randomly selected for nucleotide sequencing; the sequence obtained was used to create oRI11. A 1.2-kb product which hybridized with oRI3 was detected when oRI11 was used for PCR with oRI1 (data not shown), a primer derived from the *flaA* sequence downstream of both oRI3 and oRI9. No PCR product was detected when oRI1 was used with oRI12, a primer complementary to oRI11. Since approximately 0.3 kb would encode the N terminus (including a leader peptide) and the promoter, this product was of sufficient size to contain all of the desired upstream portion of *flaA*.

Oligonucleotides oRI1 and oRI11, with oRI11 in excess, were used in asymmetric PCR of chromosomal DNA to generate single-stranded DNA. Nucleotide sequence obtained from the product by using oRI9 as the primer was combined with that previously reported (33) to provide the sequence of the entire *flaA* gene. Three other PCR-derived products containing the 5' end of the open reading frame of *flaA* had nucleotide sequences identical to those obtained from the asymmetric PCR product (data not shown).

The *flaA* gene contains a consensus *E. coli* promoter (30) and a ribosomal binding site (RBS) (65) (Fig. 3). A 20-amino-acid signal sequence, including a typical signal peptidase I cleavage site (6) (Fig. 3, arrow), immediately precedes the N terminus previously determined by N-terminal sequence analysis of the native antigen (8, 33, 48). The precursor protein (pre-FlaA) has a molecular weight of 38,860. The deduced amino acid sequence of the first 21 amino acids of the mature protein matches precisely that of the native FlaA (8, 33, 48).

Expression of FlaA in *E. coli*. It was assumed that the native treponemal promoter would have to be substituted with a controllable *E. coli* promoter to express FlaA in *E. coli*. PCR was the most convenient method for obtaining a clonable fragment containing *flaA* exclusive of its promoter sequences. Three oligonucleotides, oRI7, oRI17, and oRI18, were synthesized for these experiments. oRI7 is complementary to a region downstream of the *flaA* termination codon; it also contains a *Pst*I site near its 5' end. oRI17 and oRI18 were derived from the sense strand of *flaA* beginning 3 and

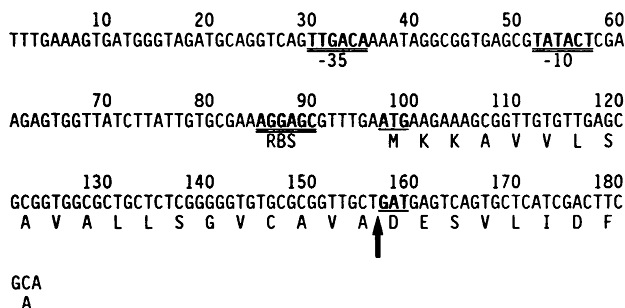

FIG. 3. Nucleotide and deduced amino acid sequences of the promoter and N-terminal coding region of *flaA*. Putative −35 and −10 *E. coli* promoter and RBS sequences are indicated by double underlining. The start codon and the codon of the first amino acid of the mature protein are indicated by single underlining. The signal peptidase I cleavage site is indicated by the arrow. The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases and assigned the accession no. M27915.

30 base pairs upstream of the RBS, respectively. oRI18 also contains two mismatches near its 5′ end which overlaps the −10 promoter sequence; these were included to ensure inactivation of the native promoter. PCR experiments with *T. pallidum* chromosomal DNA with oRI7 and either oRI17 or oRI18 produced the predicted 1.2-kb products (data not shown). Phosphorylated, purified PCR product digested with *Pst*I was ligated into pBluescript II KS– digested with *Sma*I and *Pst*I. Plasmids pRI17 and pRI18, which contain *flaA* in the correct orientation for expression from the T7 promoter, were identified by restriction endonuclease mapping and Southern hybridization with oRI3 (data not shown).

Each of these plasmids was used to transform HB101 (pGP1-2) so that expression would be temperature inducible (69). After induction, immunoblot analysis with monoclonal antibody H9-2, a murine monoclonal antibody specific for the sheath protein (33, 40), identified a doublet composed of

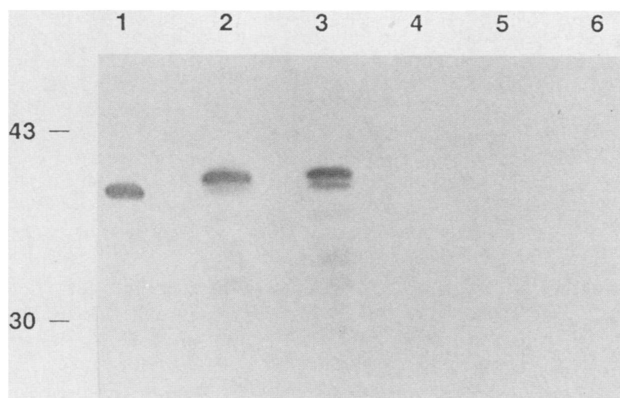

FIG. 4. Antigenic analysis of recombinant clones expressing FlaA. Western blot analysis was performed after sequential incubation in monoclonal antibody H9-2 and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G. Recombinant clones were either expressed as described in Materials and Methods (lanes 2, 3, and 4) or grown at the nonpermissive temperature (lanes 5 and 6). Lanes: 1, *T. pallidum*; 2 and 5, HB101(pRI18)(pGP1-2); 3, HB101(pRI19)(pGP1-2); 4 and 6, HB101(pBluescript II KS−)(pGP1-2). Samples were electrophoresed on an SDS-PAGE (10% polyacrylamide) gel. Molecular weight markers in kilodaltons are indicated on the left.

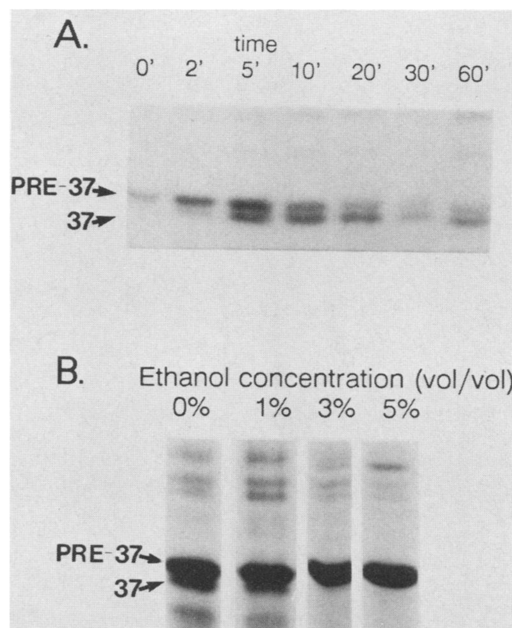

FIG. 5. Processing of FlaA in *E. coli*. The 37-kDa sheath protein (protein 37) and its precursor (PRE-37) are indicated on the left. (A) HB101(pRI18)(pGP1-2) were pulsed with Trans ^{35}S label for 1 min and then chased with 0.1% (wt/vol) methionine. Samples were removed at the indicated times, electrophoresed on an SDS-PAGE (10% polyacrylamide) gel, and subjected to autoradiography. (B) ORN103(pRI18)(pGP1-2) minicells were labeled with Trans ^{35}S label in the presence of different concentrations of ethanol, electrophoresed on an SDS-PAGE (10% polyacrylamide) gel, and subjected to autoradiography. Ethanol concentrations are shown above each lane.

39- and 37-kDa proteins in *E. coli* HB101(pRI18)(pGP1-2) (Fig. 4, lane 2) but not in HB101(pRI17)(pGP1-2) (data not shown). No H9-2 immunoreactive material was detected when the cultures were grown only at the nonpermissive temperature (Fig. 4, lanes 5 and 6). Corresponding bands were not readily seen on Coomassie blue-stained gels (data not shown).

FlaA is processed inefficiently in *E. coli*. In pulse-chase experiments with HB101(pRI18)(pGP1-2), FlaA was synthesized as a 39-kDa precursor which was subsequently processed to the 37-kDa mature protein (Fig. 5A); even after a 60-min chase, a significant amount of precursor was still present (Fig. 5A). In a parallel experiment with HB101(pT7-3)(pGP1-2) incubated under identical conditions, virtually no β-lactamase precursor was detected at 60 min (data not shown). Ethanol, a nonspecific inhibitor of membrane translocation by secretory proteins (54), was used in experiments with ORN103(pRI18)(pGP1-2) minicells. Although substantial accumulation of precursor occurred even in the absence of ethanol, inhibition of processing was ethanol concentration dependent (Fig. 5B).

Comparative 2D-PAGE analysis of recombinant and native FlaA. Because *Taq* polymerase lacks proofreading function, misincorporation of nucleotides may occur during PCR (20, 22, 60). For this reason, 2D-PAGE immunoblotting was used to confirm that the native and recombinant FlaA proteins were identical and that the 39-kDa H9-2-reactive recombinant protein was the pre-FlaA. First, ^{35}S-labeled HB101 (pRI18)(pGP1-2) was coelectrophoresed with isolated *T. pallidum* endoflagella. Immunoblot analysis with monoclo-

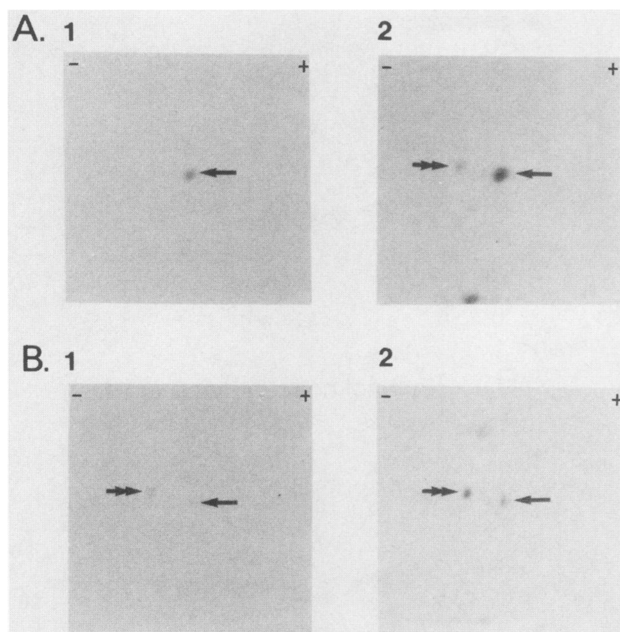

FIG. 6. Two-dimensional electrophoretic analyses of recombinant FlaA. Samples were separated by isoelectric focusing in the first dimension and by SDS-PAGE on a 10% gel in the second dimension. After transfer to nitrocellulose, the blots were incubated sequentially with H9-2 and peroxidase-conjugated rabbit anti-mouse immunoglobulin (panel 1) and then were subjected to autoradiography (panel 2). FlaA (single arrow) and pre-FlaA (double arrow) are indicated. The basic and acidic ends of the isoelectric focusing gel are indicated by − and +, respectively. (A) ^{35}S-labeled HB101 (pRI18)(pGP1-2) coelectrophoresed with isolated *T. pallidum* endoflagella. (B) ^{35}S-labeled HB101(pRI18)(pGP1-2) coelectrophoresed with hyperexpressed HB101(pRI18)(pGP1-2).

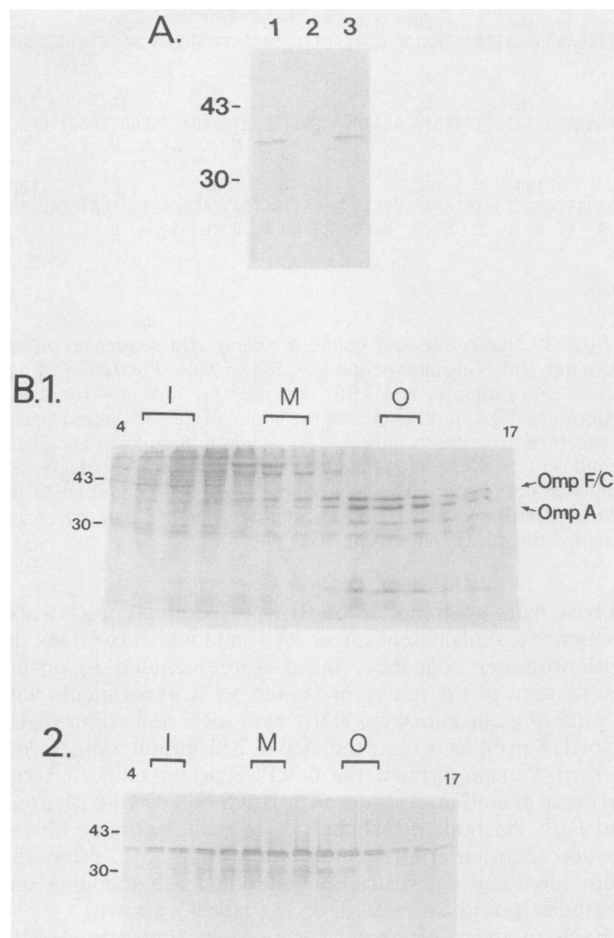

FIG. 7. Fractionation of *E. coli* clones expressing FlaA. (A) Immunoblot analysis with monoclonal antibody H9-2 of soluble periplasmic proteins released from HB101(pRI18)(pGP1-2) spheroplasts. Lanes: 1, starting material; 2, supernatant following osmotic shock of spheroplasts; 3, insoluble pellet. Samples were analyzed on an SDS-PAGE (12.5% polyacrylamide) gel. Molecular weight markers in kilodaltons are indicated on the left. (B) Coomassie blue-stained SDS-PAGE (12.5% polyacrylamide) gel (panel 1) and corresponding immunoblot probed with H9-2 (panel 2) of cell envelopes of HB101(pRI18)(pGP1-2) separated by sucrose density gradient centrifugation into inner (I) and outer (O) membranes and an intermediate membrane fraction (M). Fractions 4 to 17 are shown. Molecular weight markers in kilodaltons are indicated on the left. The *E. coli* outer membrane proteins, OmpA and OmpF/C, are indicated.

nal antibody H9-2 demonstrated that the native 37-kDa antigen (Fig. 6A, panel 1) comigrated with the 37-kDa radiolabeled protein (Fig. 6A, panel 2). In a second experiment, radiolabeled HB101(pRI18)(pGP1-2) from processing experiments was coelectrophoresed with unlabeled HB101 (pRI18)(pGP1-2) whole-cell lysates (Fig. 6B). As predicted by the presence of two lysines in the signal sequence, the pre-FlaA was significantly more basic than the mature protein during isoelectric focusing (Fig. 6A, panel 2, double arrow). The radiolabeled pre-FlaA (Fig. 6B, panel 2) comigrated with the H9-2-reactive 39-kDa antigen identified by immunoblotting (Fig. 6B, panel 1).

Construction of pRI19. Despite the above results from 2D-PAGE, the possibility still existed that the recombinant antigen contained an amino acid substitution which did not affect its pI. Such a substitution(s) could adversely impact future structure-function analyses of FlaA. As an alternative to sequencing the entire insert of pRI18 for comparison with the known sequence, an additional chimeric plasmid was subsequently constructed from fragments of known nucleotide sequence. Nucleotide sequence analysis of pRI18 showed that the 5′ sequence matched the expected sequence up to the *Eco*RI site (data not shown). pRI19 was constructed in pBluescript II KS− from the 0.3-kb *Bam*HI-*Eco*RI fragment of pRI18 (which encodes the RBS, the leader and amino acids 1 through 62 of the mature protein), and the 1.5-kb *Eco*RI-*Pst*I fragment of pRI4.3 (which encodes the remainder of the mature protein). Immunoblots of SDS-polyacrylamide (Fig. 4, lane 3) and 2D-polyacrylamide (data not shown) gels showed that HB101(pRI19)(pGP1-2) produced a H9-2-reactive 39- and 37-kDa doublet identical to that of HB101(pRI18)(pGP1-2). Processing of pre-FlaA in HB101(pRI19)(pGP1-2) was also incomplete (data not shown).

Localization of recombinant FlaA in *E. coli*. Spheroplasts derived from HB101(pRI18)(pGP1-2) were osmotically shocked to release selectively soluble periplasmic proteins. All of the FlaA remained associated with the cell pellet (Fig. 7A), whereas β-lactamase was easily detectable in the supernatant (data not shown). In a separate experiment, HB101(pRI18)(pGP1-2) whole cells were disrupted in a French press and membranes were collected by differential ultracentrifugation. The supernatant, comprising the periplasmic and cytoplasmic fractions, did not contain H9-2-reactive material (data not shown). SDS-PAGE of the

membranes fractionated on a sucrose gradient revealed protein profiles characteristic of *E. coli* inner and outer membranes (Fig. 7B, panel 1). The buoyant densities of the outer and inner membrane fractions were 1.230 g/ml and 1.167 g/ml, respectively. Immunoblot analysis of the gradient fractions (Fig. 7B, panel 2) revealed the surprising result that both the precursor and the mature protein were present in all fractions. Fractionation studies with HB101(pRI19) (pGP1-2) showed identical results (data not shown).

Diminished growth of *E. coli* clones expressing FlaA. Growth curves of HB101(pRI18)(pGP1-2) and HB101(pRI19) (pGP1-2) were compared to that of HB101(pBluescript II KS−)(pGP1-2). At the permissive temperature, a small difference in the growth rates for both HB101(pRI18)(pGP1-2) and HB101(pRI19)(pGP1-2) was consistently observed (data not shown). Viable colonies were produced from samples taken at all time points (data not shown).

DISCUSSION

For nearly a decade, recombinant DNA methodologies have been used to clone *T. pallidum* proteins in *E. coli* (68). Virtually all of these proteins were identified in genomic libraries by using monoclonal or polyclonal antibodies (68). It is clear, however, that only a limited number of recombinant *T. pallidum* proteins can be identified by using techniques dependent upon expression from native treponemal promoters. The difficulties experienced by a number of investigators in cloning the endoflagellar proteins (3, 33, 53, 74) have emphasized the need to employ alternative strategies. Pallesen and Hindersson (53) used degenerate oligonucleotide probes derived from the N-terminal amino acid sequence of the endoflagellar core proteins to identify a gene, designated *flaB2*. DNA sequence analysis revealed that *flaB2* lacked a consensus promoter, a finding which most likely explained the lack of its expression in *E. coli*.

Cloning of the 37-kDa endoflagellar sheath protein (33) was particularly problematic. Exhaustive attempts to clone the entire gene or any portion that contained the putative promoter and the N terminus were unsuccessful. A chimeric plasmid, pRI4, encoding all but the N-terminal nine amino acids of FlaA, was identified by screening a library with a nondegenerate DNA probe derived from a λgt11 clone expressing an epitope of the protein (33). To complete the nucleotide sequence of *flaA*, a strategy based on inverse-PCR was employed. Initially, we planned to use the inverse-PCR product as a template for nucleotide sequence analysis. However, a product was detected in only a few experiments and could not be used as a template for subsequent PCR reactions. Complementarity between oRI9 and oRI10 (Fig. 1), with resultant primer dimer formation during PCR (60) and/or variability in circularization of the chromosomal DNA may have contributed to this problem. Our inability to clone the relevant portion of the inverse-PCR product was not surprising in view of previous failures to clone the upstream regions of *flaA* (33). By using a sequence derived from a *Sau*3A I fragment cloned from the inverse-PCR product, the complete nucleotide sequence of *flaA* was finally obtained by asymmetric PCR.

The inability to clone putative bacterial virulence factors in *E. coli* is not unique to FlaA. Examples include the porin proteins of *Haemophilus influenzae* (29, 45) and *Neisseria gonorrhoeae* (12, 21), both of which are presumed to be toxic when expressed in *E. coli* from their native promoters. Nucleotide sequence analysis of such putatively toxic proteins often has been accomplished by generating overlapping clones, one of which encoded the putative promoter and N terminus (12, 21, 45). It is noteworthy that the nucleotide sequence of *flaA* could not be completed in this manner (33). The strategy we employed will be useful for obtaining sequences from other unclonable portions of genes.

Expression of FlaA in *E. coli* was achieved by using PCR-derived constructs in a temperature-inducible T7 expression system (69). The insert of the first construct, pRI17, encoded for only three base pairs upstream of the RBS and did not express FlaA. Analysis of the mRNA transcript suggested that a stem-loop could form between the *Sma*I site of the vector polylinker and the RBS of *flaA*, leading to inactivation of the RBS. Expression was achieved with an alternative construct, pRI18, whose insert encoded for 30 base pairs upstream of the RBS.

Nucleotide sequence analysis indicated that mature FlaA arises from cleavage of a signal peptide. Immunoblot analysis with monoclonal antibody H9-2 revealed a 37- and 39-kDa doublet in the *E. coli* transformants. The 37-kDa protein comigrated with the native FlaA by both SDS-PAGE and 2D-PAGE analyses; similar analyses with ^{35}S-labeled pre-FlaA confirmed that the 39-kDa antigen was the accumulated precursor. Although the reason(s) for inefficient processing is unclear, several possibilities were excluded. First, it is unlikely that the temperature shock required for FlaA expression contributed to the abnormal processing. In the pulse-chase experiments, bacteria were induced at 42°C for 25 min, equilibrated at 30°C prior to the addition of the radiolabel, and then chased at the same temperature. Further, β-lactamase in controls was efficiently processed under the same conditions. Second, although the amino acid sequence of the signal peptide was derived entirely from PCR products, PCR conditions associated with the lowest misincorporation rates were chosen to minimize the possibility of errors introduced by the infidelity of *Taq* polymerase (20, 22, 60). In addition, the nucleotide sequences of the signal peptides of several PCR-derived constructs were identical. Third, pre-FlaA also accumulated when expressed from pRI19, indicating that unidentified mutations downstream of the cleavage site in pRI18 were not responsible for the inefficient processing. Finally, the observations that the cleavage site so closely matches the consensus site for *E. coli* signal peptidase I (6) and that the native and recombinant FlaA had identical electrophoretic mobilities strongly argue against the possibility that a difference in cleavage site specificity exists between the *E. coli* and *T. pallidum* signal peptidases.

Overloading of the *E. coli* export machinery is the most likely explanation for inefficient processing of FlaA. This phenomenon is well recognized in *E. coli* clones hyperexpressing native *E. coli* secretory proteins (1, 2, 34, 43, 52). Analyses of other cloned treponemal secretory proteins further support this contention. The treponemal basic membrane protein (16), TmpB (28), and the 34-kDa protein (68) are all processed slowly in *E. coli*, and precursors for both TmpA and TmpB accumulate when they are hyperexpressed in *E. coli* (28, 64). In combination with the FlaA processing data, these findings suggest that *T. pallidum* secretory proteins are, in general, inefficiently processed in *E. coli*. One might speculate that toxicity resulting from progressive accumulation of pre-FlaA during unregulated expression explains our inability to clone *flaA* with its native promoter. The diminished growth of *E. coli* clones expressing FlaA at the relatively low levels achieved in our study supports this contention, although there are alternative explanations for

this finding (e.g., the increased metabolic burden of transcription).

The processing experiments indicated that recombinant FlaA should be detectable within the periplasmic space. However, FlaA was not detected in the supernatants from either osmotically shocked spheroplasts or disrupted whole cells. Instead, both precursor and mature protein were found in association with the inner and outer membrane fractions. FlaA is likely to be present as a polymer in *T. pallidum* but not so in *E. coli*. Therefore, recombinant FlaA may be relatively insoluble and have aggregated onto both the inner and outer membranes following either translocation and processing or cell disruption; proteins are not released during osmotic shock and/or spheroplasting unless they are soluble within the periplasmic space (7). Unexported pre-FlaA that accumulated within the cytoplasm most likely aggregated onto the membranes after cell disruption (72).

A number of cloned *T. pallidum* proteins have been localized putatively to the outer membrane in *E. coli* by use of methodologies similar to those employed in this study (14, 18, 28, 64, 68). These data have often been used to support an outer membrane location for the native antigens in *T. pallidum*. There are now significant reasons to question the validity of this approach. Given the ultrastructural differences between the outer membranes of *T. pallidum* and *E. coli* (59, 75), it is unclear whether location in the outer membrane of *E. coli* is relevant to the native antigen in *T. pallidum*. Furthermore, fractionation results in *E. coli* may not even be representative of the true subcellular location of the cloned antigen (72). Aberrant outer membrane localization is well recognized in *E. coli* clones expressing genetically manipulated native or hybrid secretory proteins analyzed by using the same techniques (for a review, see reference 72). The FlaA fractionation data emphasize the need for caution in extrapolating *E. coli* fractionation data to *T. pallidum*.

In *E. coli*, flagellin monomers are exported across the cell membrane by a poorly characterized, flagellum-specific mechanism which does not involve cleavage of an N-terminal signal (36). It has been proposed that monomers are transported via a channel within the growing flagellin to its distal end. The nucleotide sequences of FlaA and FlaB2 now make it possible to apply this model to the assembly of *T. pallidum* endoflagella. FlaB2 does not have a signal sequence and shares significant sequence homology with other flagellins (48, 53); presumably the endoflagellar core assembles in a manner similar to that of *E. coli* flagella. Secretion by a signal peptide-dependent pathway is consistent with the hypothesis that FlaA forms the sheath by polymerizing around the growing core. The studies reported here will provide a basis for future investigations into the genetics of endoflagella assembly and the role of the endoflagella in the pathogenesis of syphilis.

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